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Mapping of Human Chromosome Xq28 by Two-Color Fluorescence In Situ Hybridization of DNA Sequences to Interphase Cell Nuclei

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Summary

We have used the proximity of probe hybridization sites in interphase chromatin to derive the order of DNA sequences in a 2–3-Mbp region of human chromosome Xq28. The map generated bridges the results of genetic and pulsed-field gel electrophoresis mapping to produce a more complete map of Xq28 than possible with either of these other techniques alone. Two-color fluorescence in situ hybridization (FISH) was used to detect the positions of two or more probes in G_1 male interphase nuclei. We show that cosmids that are 50 kbp to 2–3 Mbp apart can be ordered rapidly with two alternative approaches: (1) by comparing the average measured distance between two probes and (2) simply by scoring the order of red and green fluorescent dots after detection of three or more probes with two fluorochromes. The validity of these approaches is demonstrated using five cosmids from a region spanning ≈800 kbp that includes the factor VIII (F8), glucose-6-phosphate dehydrogenase (G6PD), and color-vision pigment (CV) genes. The cosmid map derived from interphase mapping is consistent with the map determined by restriction-fragment analysis. The two interphase mapping approaches were then used (1) to orient the F8/CV cluster relative to two markers, c1A1 and st14c, which we show by metaphase mapping to be proximal to the F8/CV cluster, (2) to position st14c (DXS52) between c1A1 and F8, and (3) to orient the CV gene cluster relative to G6PD by using two CV-flanking cosmids, 18b41 and fr7. The probe order in Xq28 derived from interphase proximity is cen–c1A1–st14c–5′F8 (p624–p542–p625)–G6PD–18b41–3′ green–green–red–fr7–tel. We also show that, to determine their order by using metaphase chromosomes, sequences must be at least 1 Mbp apart, an order of magnitude greater than required in interphase chromatin. The data show that FISH mapping is a simple way to order sequences separated by ⩾50 kbp for the construction of long-range maps of mammalian genomes.

Introduction

The sites of sequences separated by as little as 50 kbp can be resolved in interphase nuclei by using fluorescence in situ hybridization (FISH; Lawrence et al. 1988; Trask et al. 1989). With probes from a 250-kbp region surrounding the DHFR gene in Chinese hamster cells, it was previously shown that the relative distance between DNA sequence probes in interphase is related in a simple way to the distance between the sequences on the linear DNA molecule (Trask et al. 1989). The order of seven cosmids from this region could be determined from the relative distances between pairs of probes. This approach is called "interphase mapping."

Unlike genetic mapping, interphase mapping does not depend on the availability of polymorphic markers or large pedigrees. Unlike physical mapping by pulsed-field gel electrophoresis (PFGE) of DNA cut with rare-cutting restriction enzymes, interphase mapping does not depend on the presence or methylation status of rare restriction sites. It is also not hampered by a high frequency of CpG islands, which may make it difficult

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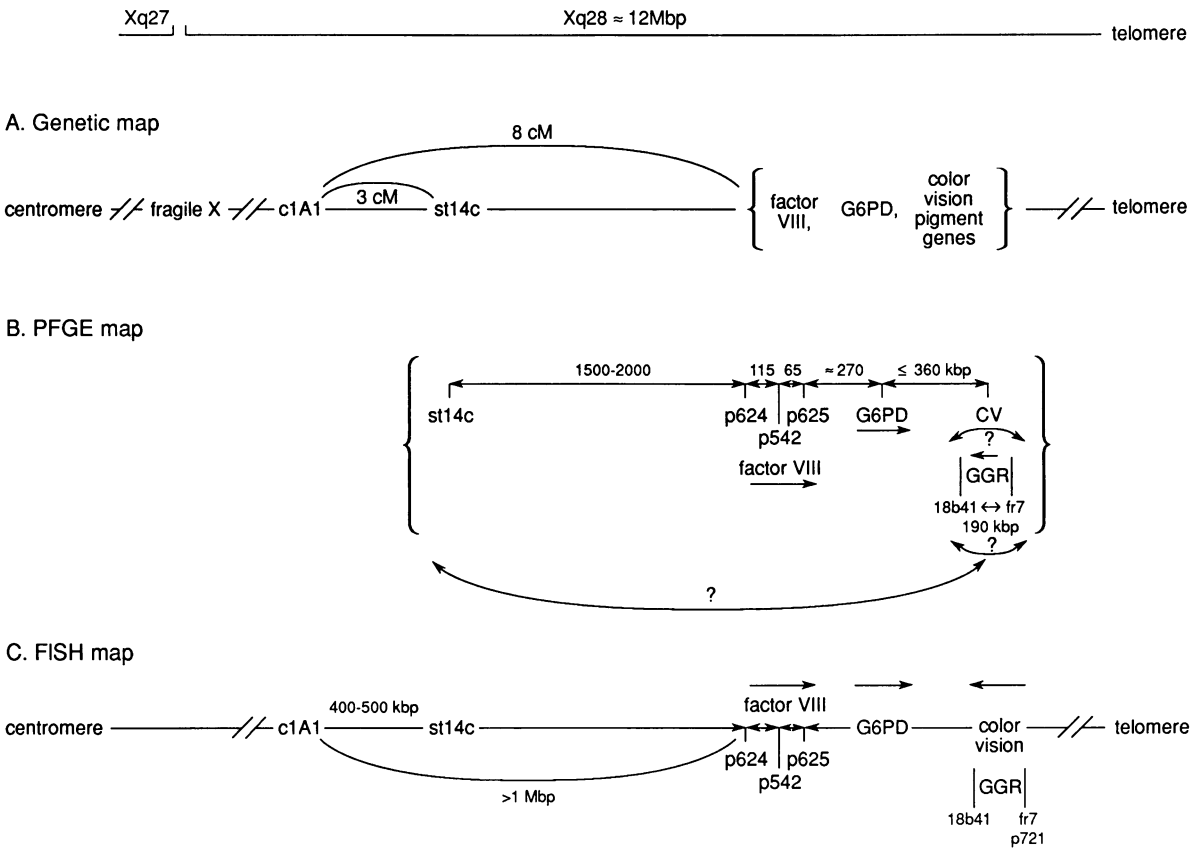

Figure 1 Maps of human chromosome Xq28. *A*, Genetic map (in cM) (from Patterson et al. 1989*a*, 1989*b*) based on the frequency of recombination between loci. *B*, Physical (PFGE) map (from Kenwrick and Gitschier 1989; Feil et al. 1990*a*). Maximal linear distances (in kbp) between probes linked by restriction digestion and PFGE or by cosmid walking. G and R denote green and red CV genes, respectively. ? = Orientation unknown. *C*, Map derived from proximity of hybridization sites in interphase nuclei. The orientation of the interphase map was accomplished by two-color metaphase mapping using c1A1 or st14 and probes from the F8/CV locus (see Results and Discussion).

to perform limited restriction digestion to link probes on large fragments. For these reasons, interphase mapping may supplement the results of genetic and physical analyses to produce genomic maps of chromosomal subregions.

Many disease loci have been localized to human chromosome Xq28 (Mandel et al. 1989), yet the map of this region is incomplete despite progress using genetic and PFGE approaches. Genes have been cloned for three loci—hemophilia A (factor VIII [F8]; Gitschier et al. 1984), glucose-6-phosphate dehydrogenase deficiency (G6PD; Persico et al. 1986), and protan/deutan color-blindness (red and green color vision pigments [CV]; Nathans et al. 1986). Candidate genes have not been identified yet for other Xq28-linked disorders. These include adrenoleukodystrophy (ALD; Aubourg et al. 1987), a form of spastic paraplegia (Kenwrick et al. 1986), dyskeratosis congenita (Connor et al. 1986), nephrogenic diabetes insipidus (Knoers et al. 1988), X-linked manic depression (Baron et al. 1987), and Emery-Dreifuss muscular dystrophy (Boswinkel et al. 1985).

Genetic and PFGE maps of Xq28 showing features relevant to this study are given in figure 1. The largest of the clusters of probes mapped by PFGE in Xq28 covers ≤3 Mbp and includes the F8, G6PD, and CV genes (Gitschier et al. 1984; Patterson et al. 1987, 1988; Arveiler et al. 1989; Kenwrick and Gitschier 1989). Physical linkage between F8 and st14c (DXS52) has been shown by PFGE, but only for a single individual (Kenwrick and Gitschier 1989). The physical distance between c1A1 (DXS374) and st14c is unknown, since they

have not yet been physically linked. Genetic analysis indicates that c1A1 is proximal to both DXS52 and the F8/CV cluster (3 and 8 cM, respectively) (Patterson et al. 1989*a*). Three multiply informative recombination events place the F8/CV cluster distal to DXS52 (Patterson et al. 1989*b*). While these data can be used to infer the order centromere–c1A1–st14c (DXS52)– F8–G6PD–CV–telomere, this order requires confirmation.

Here we report application of interphase mapping to determine the order of probes that span 2–3 Mbp in Xq28. We describe two alternative approaches to determine cosmid order by FISH mapping in interphase nuclei. (1) Order can be derived by comparing the relative distance measured between two probes. The order ABC is inferred if the measured distance A-C is greater than both the distances A-B and B-C. The two probes can be labeled with different colors to ensure that both have hybridized to each nucleus analyzed. (2) Order can be derived from the order of red and green fluorescent dots in nuclei that have been hybridized with three or more probes simultaneously. For example, two probes can be detected in red and a third probe in green. The position of the green site either between the two red sites (R-G-R) or outside the two red sites (G-R-R) is then scored to determine probe order. We first demonstrate the validity of these approaches by using five probes in Xq28 from the 810-kbp F8/G6PD/CV cluster whose relative positions within the cluster have been determined by PFGE analysis (Kenwrick and Gitschier 1989). Further interphase analyses are presented that predict the following order for Xq28 probes: cen–c1A1–st14c–F8–G6PD–CV–telomere. We also establish the orientation of the CV gene cluster relative to G6PD. We conclude that interphase mapping by FISH is an efficient tool for constructing long-range maps of mammalian genomes.

Material and Methods

Interphase Nuclei Preparation

A foreskin fibroblast culture (HSF7) from a normal male was held at confluency without medium change for 4 d to enrich for G_1 interphase cells. Cells were collected by trypsinization, washed twice in PBS/0.1% BSA, swollen in 75 mM KCl at 37°C for 15 min, fixed in three changes of 3:1 methanol:acetic acid, and dropped on glass slides.

Metaphase Spreads

Metaphase spreads were prepared from PHA-stimulated peripheral blood lymphocytes of a healthy male, according to a method described by Harper et al. (1981). Cells were synchronized by methotrexate block and thymidine release, collected after a 15-min exposure to colcemid, and then swollen and fixed as described above.

Probes

Nine cosmid probes were used. p624, p625, and p542 contain different portions of the F8 gene in pGcos4 vector (Gitschier et al. 1984). G6PD and CV were selected for homology to the G6PD gene and to the red and green pigment genes, respectively, from a human cosmid library in pwe15 (Stratagene) by using probes provided by M. G. Persico and J. Nathans (J. Gitschier, unpublished results). c1A1 represents the DXS374 locus (Patterson et al. 1989*a*) and was provided by K. E. Davies. 18b41 and fr7 contain sequences flanking the CV genes (Feil et al. 1990*a*). Two probes were employed to mark the position of the DXS52 locus: c33A3-1, a cosmid containing 43 kbp encompassing the st14c and DXS134 loci (Feil et al. 1990*b*), and st14c, a plasmid containing a 9-kbp genomic fragment at the st14c locus (Oberlé et al. 1985). Members of the st14 family are dispersed over a 600-kbp region in Xq28, and both c33A3-1 and st14c map ≈400 kbp from the VNTR region at the DXS52 locus that is often used for genetic analyses (Feil et al. 1990*b*). Fr7, 18b4-1, c33A3-1, and st14c were provided by J.-L. Mandel and R. Feil. Plasmid subclone p721 contains 9 kb of sequence 5′ of and not including the red cone pigment gene (S. Kenwrick, unpublished results). Purified cosmid or plasmid DNA was labeled with either biotin-11-dUTP (Enzo) or digoxigenin-11-dUTP (Boehringer-Mannheim), via nick translation using a commercially available kit (BRL) and twice the recommended amount of labeled nucleotide.

Hybridization and Detection

Hybridization conditions were as described elsewhere (Trask et al. 1989), with a few modifications. Ten microliters of a hybridization mixture containing a total of 20 ng mixed probes (divided equally among two or more probes), 1 μg human sonicated DNA (to suppress hybridization of repetitive sequences in the probes to the target), 50% formamide, 2 × SSC, and 10% dextran sulfate at pH 7 were denatured at 70°C for 5 min and incubated at 37°C for 45 min before being applied to each denatured slide. Slides were incubated ≥16 h at 37°C. After posthybridization washing, slides hybridized only to biotinylated probes were incubated in avidin-fluorescein as described elsewhere (Trask et

al. 1989). Slides hybridized to both biotinylated and digoxigenin-labeled probes were treated at room temperature as follows: (1) one 5-min wash in 4 × SSC, (2) 5-min preblock in 4 × SSC/0.1% BSA, (3) 1-h incubation in 2.5 μg avidin–Texas red (Vector Laboratories)/ml in 4 × SSC/0.1% BSA (buffer as described by Lawrence et al. 1988), (4) one 10-min wash in each of 4 × SSC, 4 × SCC/0.1% Triton X-100, 4 × SSC, and PN (0.1 M phosphate buffer at pH 8.0 containing 0.1% NP-40), (5) 5-min preblock in PNM (PN containing 5% nonfat dry milk and 0.01% sodium azide, spun 15 min at 200 *g* to remove milk solids), (6) 1-h incubation in 15 μg sheep anti-digoxigenin Fab fragments (Boehringer Mannheim)/ml and 5 μg biotinylated goat anti-avidin (Vector)/ml in PNM, (7) three 10-min washes in PN, (8) 5-min preblock in PNM, (9) 1-h incubation in 1:50 dilution of FITC-conjugated rabbit anti-sheep IgG (Vector) and 2.5 μg avidin–Texas red/ml PNM, and (10) three 10-min washes in PN. An antifade solution containing 0.1 μg DAPI/ml was applied to all slides before viewing.

Hybridization-Site Analysis

Slides were coded before being viewed with a Zeiss Plan Neofluor oil immersion objective (100 ×; 1.3 numerical aperture) on a Zeiss Axio-series epifluorescence microscope. In some cases, a 1.25 × optivar was used. Epifluorescence filters for slides labeled with FITC only were as follows: excitation, BP485 nm; reflector, 510 nm; and emission, LP520. A double-band pass filter (Omega, Brattleboro, VT) was used to view FITC and Texas red fluorescence simultaneously. In this filter, excitation band-pass filters are centered around 490 nm and 560 nm; and dichroic and emission band-pass filters are centered around 530 nm and 650 nm. Photographs were taken using Ektachrome 400ASA color slide film. Distances between hybridization sites were determined as described elsewhere (Trask et al. 1989). In other experiments, three or more probes were hybridized simultaneously to nuclei and were detected in two colors to determine probe order. Nuclei to be scored were selected only on the basis that they showed two hybridization sites in one color and a third in the second color. To determine probe order along metaphase chromosomes, two probes were hybridized simultaneously and were detected in different colors. Randomly selected chromatids showing both red and green fluorescent labels were scored visually. The position of one probe relative to the other along a line parallel with the long axis of the chromosome was scored as proximal, distal, or even (i.e., on top of, or side by side). The statistical significance of interphase and metaphase results was determined by calculating the test statistic, $z = 2\sqrt{n}(f - .5)$, where n is the number of observations and where f is the observed fraction of observations in one of two classes, and comparing z with tables of the normal distribution to obtain two-tailed P values.

Results

Probe Order Can be Derived from Interphase Distance

One indication that interphase distance (in μm) is correlated to genomic distance (in kbp) was obtained by visually comparing (*a*) nuclei hybridized with probes known to be separated by 450–630 kbp (CV and p625) (fig. 2*A*) with (*b*) nuclei hybridized with probes separated by ⩽360 kbp (CV and G6PD) (fig. 2*B*). In many of the nuclei shown, the distance between CV and p625 is noticeably greater than the distance between CV and G6PD.

Before assay of the distance between hybridization sites produced by pairs of probes, each of five cosmids from the F8/CV cluster was hybridized individually to preparations of interphase nuclei. This test revealed that individual cosmids can produce more than a single round fluorescent spot in interphase. For example, CV produces several fluorescent spots in a cluster averaging 0.3 μm in diameter (depicted in green in figs. 2*A* and 2*B*). The average person has one red and two green pigment genes arranged in a tandem array of three 40-kbp units (Nathans et al. 1986; Drummond-Borg et al. 1989). Therefore, the cluster of fluorescent spots presumably represents the multiple binding sites of CV in each nucleus and illustrates the resolution of the FISH technique. Three other cosmids from Xq28 occasionally produced two closely spaced fluorescent spots ("double dots") when hybridized alone (p625, p624, and G6PD in 15%, 19%, and 27% of nuclei, respectively). The mean ± SEM distance between the spots produced by each of these three cosmids ranged from 0.06 ± 0.02 μm (n = 105) for p625 to 0.14 ± 0.02 μm (n = 148) for G6PD and is small relative to the distance measured between two different cosmids (see below). For these probes, double dots may indicate that some nuclei in the preparations have entered S-phase and have duplicated the target site of a given cosmid. Alternatively, double dots may be an artifact of the nature of avidin accumulation at the hybridization site. The fifth probe from the F8/CV cluster, p542, produced two widely separated fluorescent dots in some experiments. This hybridization pattern is consistent with the

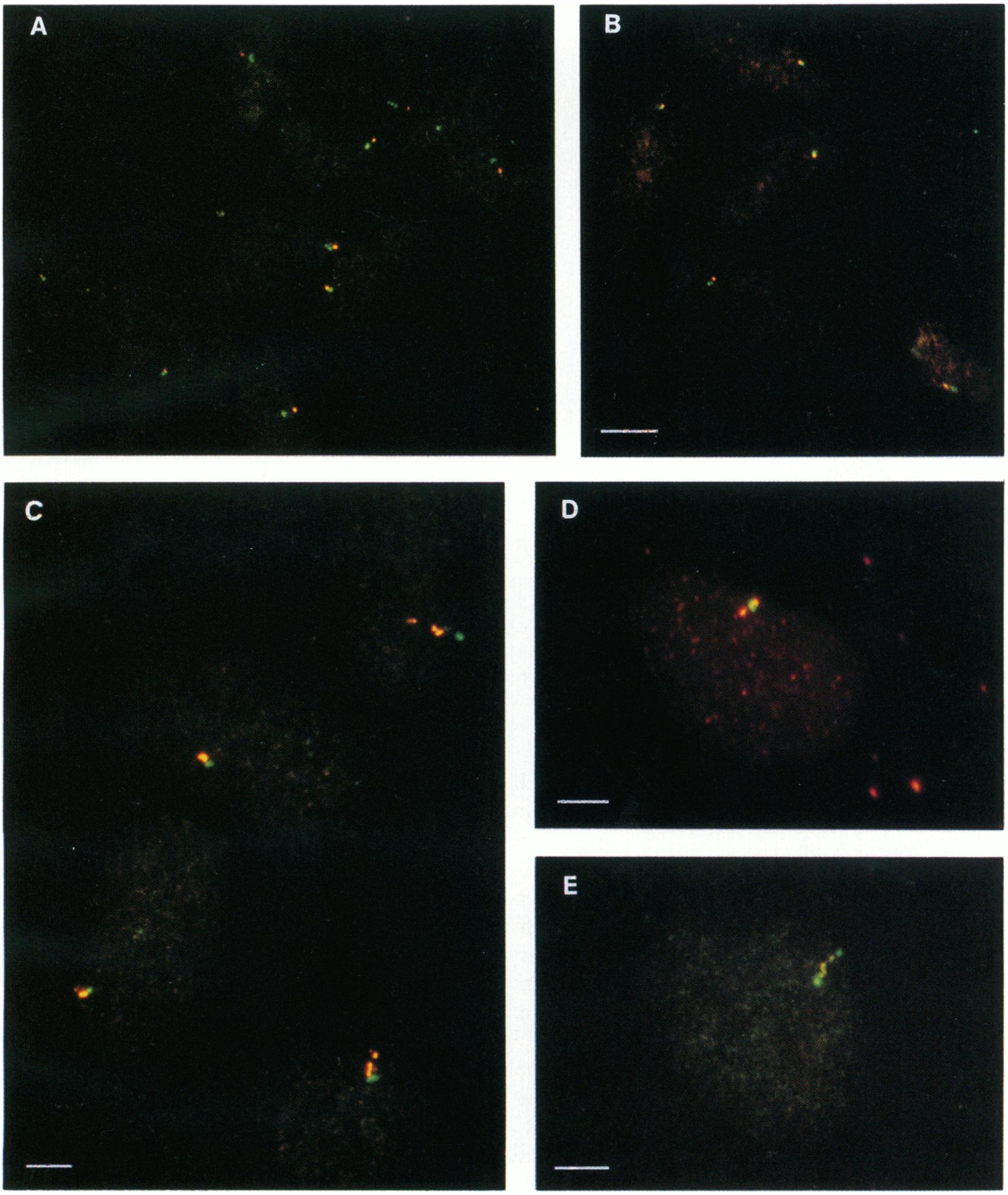

Figure 2 G_1 interphase nuclei of normal male fibroblast cells after two-color FISH with two or more Xq28 probes. Incompletely suppressed repetitive sequence hybridization allows delineation of the nuclei. Red and green fluorescence are viewed, without filter change, through a double band-pass filter. *A,* CV genes labeled with digoxigenin and detected with FITC-labeled antibody (note multiple hybridization sites per nucleus). p625, an F8 probe known to map 450–630 kbp from CV, was labeled with biotin, and its hybridization site was detected with avidin–Texas red. *B,* Red: G6PD, known to map within 360 kbp of CV. Green: CV genes. Scale bar for *A* and *B* = 10 μm. *C,* Red: p625, p542, p624, and G6PD. Green: CV. Scale bar = 5 μm. *D,* Green: st14-c locus marked with cosmid c33A3-1. Red: c1A1 and p624. Scale bar = 5 μm. *E,* Red: p625, p542, p624, and G6PD. (Texas red appears more orange in this reproduction.) Green: c1A1 and CV. Scale bar = 5 μm.

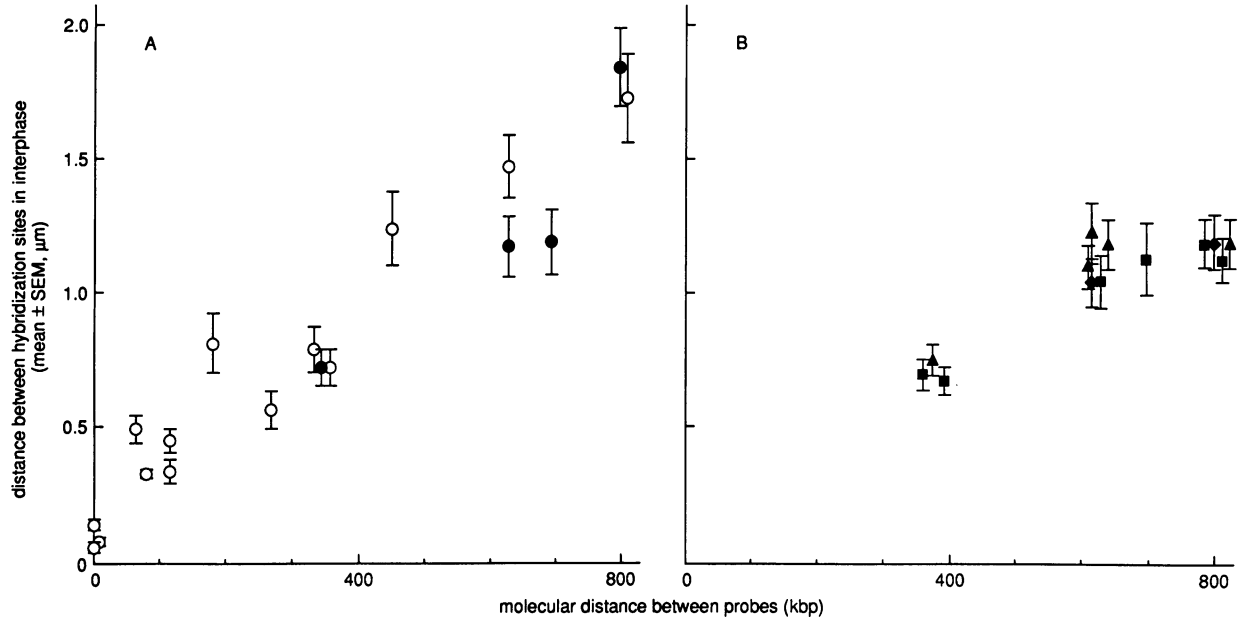

Figure 3 Relationship between measured distance between probe hybridization sites in G_1 interphase nuclei and maximal distance between midpoints of probes on linear DNA molecule, as estimated by PFGE analysis (Kenwrick and Gitschier 1989). Nuclei were hybridized simultaneously with two probes. The hybridization sites of the two probes were labeled either with different fluorochromes (blackened symbols) or with the same fluorochrome (FITC) (open symbols). Each point represents the mean ± SEM of distance measurements made on 30–60 randomly selected nuclei. Note that SEMs estimate the accuracy of the mean but do not accurately reflect 68% confidence limits of the distributions, since the distributions of the measurements are not normal. *A*, Data from nuclei preparation I. The values plotted at a distance of 0 kbp represent the average measured distance between the fluorescent dots produced by single cosmids. The measured distance between the furthest two dots in the cluster of dots produced by the CV cosmid (0.3 μm) is plotted at 80 kbp. *B*, Data from three additional preparations of interphase nuclei: II (triangles), III (diamonds), and IV (squares).

fact that p542 contains a portion of intron 22 of the F8 gene that is repeated at a second site within 1.2 Mbp of F8 (Patterson et al. 1989*b*; Levinson et al. 1990). When p542 was paired with other Xq28 probes in experiments described below, only the distance from p542's brighter and more efficiently labeled site within F8 to the site of the other probes was recorded.

Using five cosmids from the 810-kbp F8/CV region, we systematically compared the distance between probe hybridization sites in interphase with the known distance between probes on the linear molecule. Cosmids were hybridized in pairs to interphase nuclei in two types of experiment, and the distance was measured between the approximate centers of the hybridization sites produced by the two probes in each pair (fig. 3*A*). In one type of experiment, both probes in a pair were detected with FITC (fig. 3, open symbols). This has the advantage of requiring short film-exposure times (≈20 s) for image collection, but works only if the detection of hybridization sites approaches 100% efficiency. In the second type of experiment, the two probes are labeled with different fluorochromes (FITC and Texas red), as illustrated in figures 2*A* and 2*B* (fig. 3, blackened symbols). Two-color labeling has the advantage that nuclei in which both probe sites are labeled can be identified and selected for analysis. A double-band pass filter is required to view both colors simultaneously to avoid the shift in image often produced by changing filters. Use of this filter has the disadvantage that 150–200-s exposure times are needed to collect adequate images of red and green hybridization sites on film. This is 10-fold longer than the time required to collect images of nuclei labeled with FITC only.

The results in figure 3*A* show that mean interphase distance increases steadily with the genomic distance between the probes' midpoints (as determined by PFGE) to at least 800 kbp in both one- and two-color experiments. The reproducibility of this relationship was tested using three additional nuclear preparations hybridized with a limited number of cosmid pairs from the F8/CV cluster (fig. 3*B*) in two-color experiments. These experiments indicate that, up to 600 kbp, the relationship

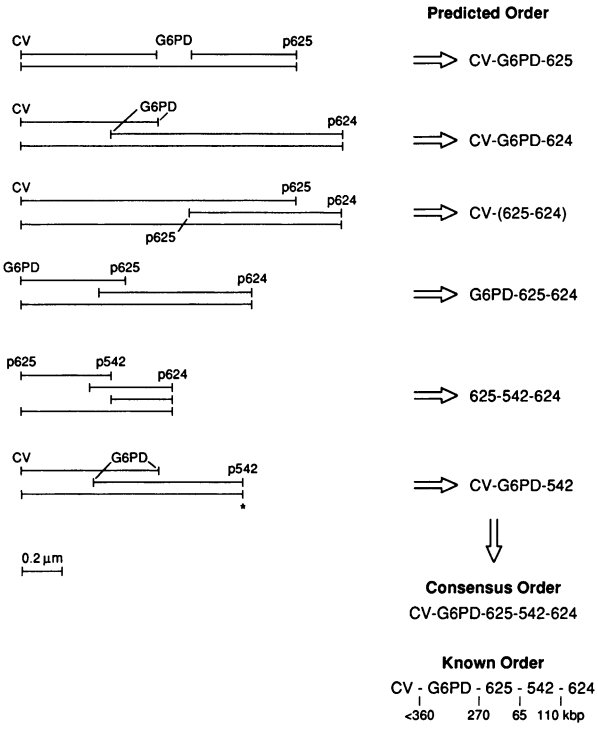

Figure 4 Probe order derived from G_1 interphase distance measurements. The lengths of the lines are proportional to the mean interphase distance measured between the indicated probes in a one-color experiment using nuclear preparation I (see fig. 3*A*, open circles).

between interphase and linear distance is approximately the same in repeated measurements and in different preparations of nuclei. However, the slope of the relationship beyond 600 kbp varies between preparation I and preparations II–IV. The variable in the preparation procedure that accounts for this difference has not been identified.

Figure 4 shows that the three possible pairwise interphase distance measurements between three probes can be used to infer probe order on the linear DNA molecule. The data shown are derived from the one-color experiment using nuclei preparation I (fig. 3*A*, open circles). For example, the distance CV-p625 is greater than both the distances CV-G6PD and G6PD-p625. Thus, we conclude that the order is CV–G6PD–p625. The order derived from the interphase distance of five cosmids within the F8/CV cluster (p624–p542–p625–G6PD–CV) is consistent with the published restriction map for this region (Kenwrick and Gitschier 1989).

Probe Order Can be Derived from the Order of Red and Green Fluorescent Dots after Two-Color Hybridization with Three or More Probes

Probe order can be determined more directly and simply by labeling the hybridization site of one probe in green and the sites of two or more other probes in red.

Table 1

Two-Color Metaphase Mapping to Determine Probe Order

Position of:	Relative to:	% of Chromatids			No. of Chromatids Scored
		Proximal	Even	Distal	
c1A1	p624 (F8)	67[a]	15	18	55
c1A1	p625 (F8)	59[a]	8	33	106[b]
c1A1	18b41	67[a]	19	14	42
c1A1	CV	64[a]	22	15	151
st14c	p624 (F8)	70[a]	3	27	107[b]
st14c	p625 (F8)	57[a]	11	31	110[b]
st14c	18b41	61[a]	10	30	135[b]
st14c	CV	61[a]	8	31	117[b]
c1A1	st14c	37	16	47	106[b]
F8[c]	CV	25	28	46[d]	183

[a] Significantly greater (two-sided *P* values vary but are less than .005) than the percentage that lies distal.

[b] Scored on metaphase chromosomes of a normal female. Remaining data are from metaphase chromosomes of a normal male. Mean ± SD length of chromosome X was 9 ± 1 µm (n = 45) in the female preparations and 10 ± 2 (n = 65) in the male preparations.

[c] Data include experiments using one of three different probes—p624, p542, or p625—representing the F8 locus.

[d] Significantly greater ($P < .001$) than the percentage that lies proximal.

The position of the green site relative to the red sites is then scored without the need to record images on film. Figure 2*C* shows an example of this approach using probes from the 810-kbp F8/CV cluster. The CV hybridization site is labeled in green. The sites of four other probes, three from F8 (p625, p542, and p625) and one for G6PD, are labeled in red. The CV hybridization site is outside the line or cluster of red hybridization sites in all nuclei shown. This outside position of the CV site was observed in 92% of 63 randomly selected nuclei hybridized with this set of probes. The CV hybridization site was between or on top of a red site in only 8% of the nuclei. In another experiment, G6PD was detected in green while two other probes, p542 and CV, were detected in red. The green spot for G6PD was between the red sites in significantly more nuclei than it was outside the red sites (28 vs. 9; $P < .005$). The order of hybridization sites in interphase observed in both experiments is consistent with the restriction map for the F8/CV cluster (Kenwrick and Gitschier 1989).

Two-Color Mapping to Metaphase Chromosomes

Two-color mapping to metaphase chromosomes showed that both c1A1 and st14c are proximal to probes from the F8/CV cluster (table 1), confirming the results of genetic analysis (Patterson et al. 1989*a*). The hybridization sites of c1A1 and st14c were proximal to the hybridization sites of probes from the F8/CV cluster in 57%–70% of chromatids scored. This fraction was significantly greater ($P < .005$) than the fraction of chromatids observed in which the hybridization sites of c1A1 or st14c lay distal to those of the F8/CV probes (14%–33%).

In contrast, c1A1 and st14c could not be ordered relative to each other with statistical confidence when metaphase analysis was used (table 1). The fraction of chromatids in which the hybridization site of st14c was distal to the hybridization site of c1A1 is not significantly different from the fraction in which st14c was proximal to c1A1. This presumably results from the relative proximity of c1A1 and st14c (see below) and from the high degree of DNA compaction in metaphase chromosomes.

We next tested whether the 600–800-kbp distance between F8 probes and CV was sufficient to allow us to orient the F8/CV cluster with respect to the telomere on metaphase chromosomes. Two X chromosomes from normal male metaphase spreads are shown in figure 5 to illustrate that F8 and CV probes can be resolved in metaphase chromosomes. The green hybridization site of CV lies proximal to the orange-red site of F8 in each of the four chromatids shown. We scored the relative positions of CV and F8 probes in 183 chromatids. The hybridization site of CV was proximal to the hybridization site of F8 probes in significantly more chromatids than it was distal ($P < .001$) (table 1). This result suggests the order cen–CV–F8–telomere. This order is inconsistent with combined genetic and physical mapping results (Kenwrick and Gitschier 1989; Patterson et al. 1989*a*), which predict the opposite orientation, cen–F8–CV–telomere. A possible explanation for this discrepancy is presented below in the Discussion.

Interphase Mapping Predicts the Order c1A1–st14c–(F8CV)

Because st14c could not be positioned on metaphase chromosomes relative to c1A1, we used interphase mapping to map st14c relative to c1A1 and the F8/CV cluster. Two different probes were used to mark the st14c locus: (1) a 43-kbp cosmid, c33A3-1, and (2) a 9-kbp plasmid, st14c. A series of experiments was performed in which the positions of three probes—c1A1, a probe from the F8/CV cluster, and either c33A3-1 or st14c—were labeled in two colors in interphase nuclei (table 2, lines 1–7). For example, the hybridization site for st14c was found 2.8-fold more frequently between than outside the pair of hybridization sites produced by c1A1 and CV (table 2, line 2). C33A3-1 was also located more frequently between than outside the sites of c1A1 and the F8 probe, p624 (fig. 2*D* and table 2, line 7). The results of all experiments predict that st14c maps in the interval between c1A1 and the F8/CV cluster, in agreement with previously published recombination frequency data (Patterson et al. 1989*a*).

Interphase Mapping Shows That the Orientation of the F8/CV Complex Is cen-F8-CV-tel

We applied interphase mapping to resolve the discrepancy between the orientation of the F8/CV complex predicted by metaphase mapping and that predicted by available genetic and PFGE mapping (Kenwrick and Gitschier 1989; Patterson et al. 1989*a*). Probes from the F8/CV cluster were ordered relative to either c1A1 or st14c in several interphase mapping experiments. Because both metaphase and genetic mapping place c1A1 and st14c proximal to the F8/CV cluster, establishing the orientation of the F8/CV cluster relative to either of these probes orients the cluster relative to the centromere.

Four experiments were conducted to order F8 and CV relative to st14c in interphase chromatin (table 2,

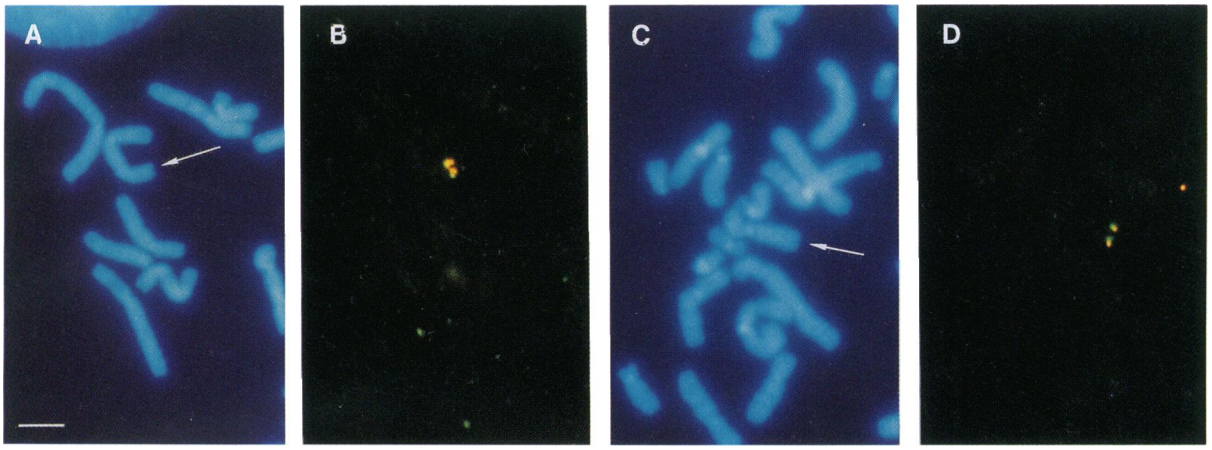

Figure 5 Portions of two metaphase cells from a normal male showing X chromosomes after two-color FISH with Xq28 probes. *A* and *B*, Green: CV; Red: probes p624, p542, p625, and G6PD. *C* and *D*, Green: CV; Red: p624. The DAPI fluorescence image is shown in panels *A* and *C*; the corresponding Texas red/FITC fluorescence image is shown in panels *B* and *D*. The yellow-green fluorescence of CV is proximal to the orange-red fluorescence of the other probes in all four X chromatids shown. Scale bar = 5 μm.

lines 8–11). In these experiments, the sites of st14c and an F8 probe (either p624 or p625) were labeled in red. The third probe (CV, G6PD, or 18b41) hybridization site was labeled in green. The position of the green probe relative to the sites of st14c and F8 was scored. The data indicate that the order st14c–F8–(G6PD, 18b41, CV) is more likely than the order st14c–(G6PD, 18b41, CV)–F8, consistent with PFGE results on one individual (Kenwrick and Gitschier 1989).

F8 and CV were also ordered relative to c1A1 in four multiprobe/two-color interphase mapping experiments. All experiments indicate that F8 is closer to c1A1 than

Table 2

Order of Hybridization Sites of Three Probes in G_1 Interphase Nuclei

Probe Order A	Ratio of Observed Probe Orders, A:B	Probe Order B	No. of Nuclei
1. (c1A1 st14c) **CV**	6.4:1[a]	c1A1 **CV** st14c	126
2. c1A1 **st14c** CV	2.8:1[a]	(c1A1 CV) **st14c**	171
3. (c1A1 st14c) **p624**	4.7:1[a]	c1A1 **p624** st14c	109
4. c1A1 **st14c** p624	2.0:1[a]	(c1A1 p624) **st14c**	197
5. **c1A1** (33A31 p624)	2.7:1[a]	33A31 **c1A1** p624	66
6. (c1A1 33A31) **p624**	3.6:1[a]	c1A1 **p624** 33A31	55
7. c1A1 **33A31** p624	2.7:1[a]	(c1A1 p624) **33A31**	88[b]
8. (st14c p624) **18b41**	2.0:1[a]	st14c **18b41** p624	209
9. (st14c p624) **CV**	1.9:1[a]	st14c **CV** p624	121
10. (st14c p624) **G6PD**	1.2:1	st14c **G6PD** p624	159
11. (st14c p625) **CV**	1.5:1[a]	st14c **CV** p624	112

NOTE.—In experiments summarized in lines 1–8 and 10, two probes were detected using Texas red and the third probe (indicated in underlined boldface) was detected using FITC. In lines 9 and 11, the probe indicated in underlined boldface was labeled in red and the other two were labeled in green. The position of the third probe either between or outside the other two probes was scored in the indicated number of nuclei. Nuclear preparation III was used in all cases. At present, we have no explanation for the 2.3-fold difference in the values obtained in lines 1 and 2 and in lines 3 and 4.

[a] Significantly different ($P < .05$) than 1:1.

[b] One nucleus from this experiment is shown in fig. 2*D*.

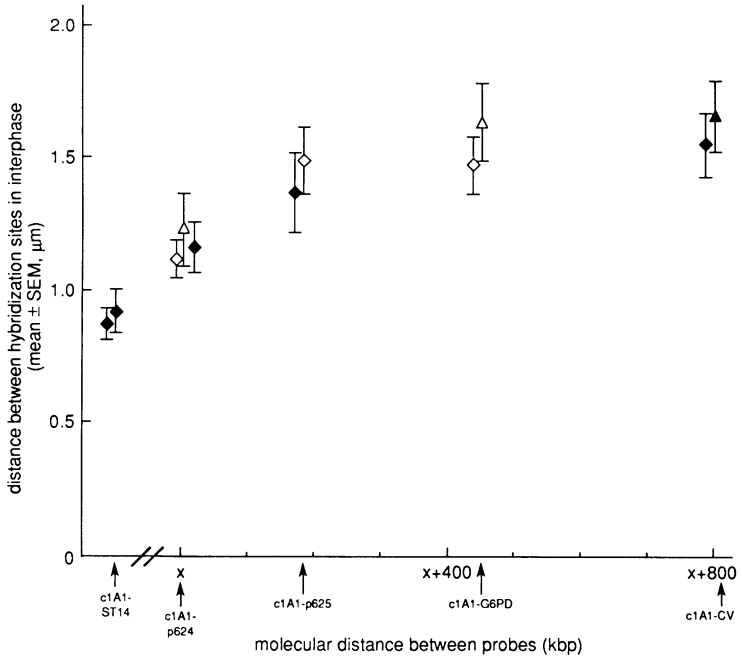

Figure 6 Measured distance in G_1 interphase nuclei between hybridization sites of c1A1 and st14c or probes from F8/CV cluster. The physical distance between c1A1 and p624 is unknown and is indicated as x kbp. The physical distance between c1A1 and CV is then (x + 810) kbp. Mean interphase distance was measured between two probes by using two sets of nuclei (II and IV) in one- and two-color experiments. The positive slope of the plotted relationship confirms the order c1A1–st14c–p624–p625–G6PD–CV. Since c1A1 is proximal to the F8/CV cluster, the relative interphase distances serve to orient the F8/CV cluster on the chromosome. See fig. 3 legend for identification of symbols.

is CV. In the first experiment, the hybridization sites of c1A1 and CV were labeled in green and those of three F8 cosmids and of G6PD were detected in red. One nucleus from this experiment is shown in figure 2*E*. CV and c1A1 appear at either ends of a line of red hybridization sites in this nucleus. We scored 22 nuclei in which the red fluorescent sites formed a line rather than a cluster. The green sites of c1A1 and CV were separated by the red hybridization sites in significantly more nuclei than they were together and not separated by red hybridization sites (82% vs. 18%, $P < .005$). This result suggests that CV is at the opposite end of the F8/CV cluster from c1A1. The same probes and labels were used in a second experiment, in which nuclei were scored regardless of the arrangement of the red sites. Again, c1A1 and CV were separated by F8 and G6PD probes in the majority of nuclei (43 of 67 scored; $P < .05$). In a third experiment, CV was the only probe labeled in green, while c1A1, the three F8 cosmids, and G6PD were labeled in red. The green CV site was found outside the cluster of red hybridization sites in significantly more nuclei than it was found between the red hybridization sites (27 vs. 14; $P < .05$). The fourth experiment was similar to the third, except that G6PD was not included in the set of red probes. Again, the green site representing the CV locus was positioned outside more frequently than it was between the red sites (23 vs. 8; $P < .01$). The last two experiments demonstrate that CV does not map between F8 and c1A1. Thus, together with the data presented in the previous sections, the order of red and green hybridization sites in interphase predicts the order cen–c1A1–st14c–p624–p542–p625–G6PD–CV–tel.

We next tested whether the F8/CV cluster could be oriented by comparing the mean interphase distance between F8/CV probes and c1A1, given the fact that together these probes span >2 Mbp. The physical distance between c1A1 and p624 is unknown but, given the data presented above, must exceed the distance between st14c and p624. St14c and p624 are 1.5–2 Mbp apart (Kenwrick and Gitschier 1989). The interphase distances measured from c1A1 to each of four probes from the 810-kbp F8/G6PD/CV cluster and to st14c are shown in figure 6. The measurements in figure 6

were made using nuclei preparations II and IV and thus extend the relationship, plotted in figure 3*B*, between interphase distance and genomic distance. The slope of the relationship in figure 6 is low, but it is positive as plotted. The measured distance between c1A1 and CV is greater than that between c1A1 and p624. The distances between c1A1 and the other probes are intermediate. Thus, interphase distance does not reach a plateau at 800 kbp, as the data in figure 3*B* suggest, but continues to increase gradually beyond 1 Mbp. These data again suggest the order cen–c1A1–p624–p625–G6PD–CV–tel and illustrate that interphase distance may be used to order sequences spanning 2–3 Mbp.

The distance measured between c1A1 and st14c is less than the distance between c1A1 and p624, confirming the position of st14c between c1A1 and p624. Because the st14c-to-c1A1 distance (≈0.9 μm) falls in the steep part of the relationship in figure 3*B*, one can use interphase distance to estimate the distance between c1A1 and st14c as 400–600 kbp.

Interphase Mapping Orients the CV Gene Complex Relative to G6PD

It is not known whether the 5′ or the 3′ end of the CV gene complex is closer to G6PD. This is important because a deletion 5′ to the red pigment gene has been identified in one individual with ALD and blue-cone monochromacy (Aubourg et al. 1990). Thus, orientation of the CV genes relative to G6PD would expedite the identification of the genetic lesion in ALD by allowing a walk toward the locus from two directions.

To orient the CV gene cluster relative to G6PD, we used the two interphase mapping approaches described above and two cosmids known to flank the CV genes. 18b41 and fr7 are ≈190 kb apart. 18b41 maps ≈50 kbp 3′ to the last green pigment gene, and fr7 maps ≈25 kbp 5′ to the red pigment gene (Feil et al. 1990*a*). The distance measured between 18b41 and G6PD was less than the distance between fr7 and G6PD in four experiments using two different preparations of nuclei (fig. 7). The distance between G6PD and CV was intermediate. This suggests the order G6PD–18b41–CV–fr7. The same order was found by determining the order of red and green dots after hybridization and detection of G6PD and fr7 in red and 18b41 in green. The green hybridization site of 18b41 appeared significantly more often between than outside those of fr7 and G6PD (72 vs. 34; $P < .0005$).

Similarly, the map position of p721 was determined from its proximity, in interphase, to G6PD, fr7, and 18b41. The hybridization site of p721 is not resolvable

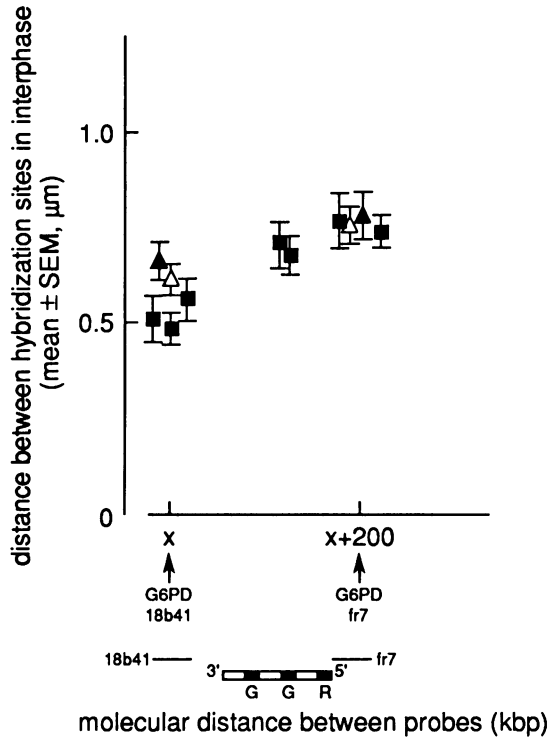

Figure 7 Measured distance in G_1 interphase nuclei between hybridization sites of G6PD and three probes in or near CV gene cluster. The physical distance between G6PD and the CV gene cluster is ⩽360 kbp (Kenwrick and Gitschier 1989). 18b41 maps ≈50 kbp 3′ of the last green pigment gene, and fr7 maps ≈25 kbp 5′ of the red pigment gene (Arveiler et al. 1989); they are ≈190 kbp apart. The linear distance between G6PD and the closest probe (here taken as 18b41) is indicated by x kbp. The distance between G6PD and the farthest of the two probes (here taken as fr7) is (x + 190) kbp. The positive slope suggests that the linear order is G6PD–18b41–3′ CV–fr7. See fig. 3 legend for identification of symbols.

from fr7, consistent with the fact that p721 contains 9kb of sequence 5′ to and not including the red cone pigment gene (S. Kenwrick, unpublished data). The hybridization site of 18b41 was found significantly more often between than outside the hybridization sites of p721 and G6PD in three-probe/two-color experiments (39 vs. 13; $P < .0005$).

These observations suggest the order G6PD–18b41–3′ green–green–red–5′–(fr7, p721). Given the data shown above, this suggests that fr7 and p721 are the most telomeric of the probes used in Xq28.

Discussion

Interphase mapping is a straightforward and relatively rapid technique for ordering DNA sequences. Our

data indicate that order can be derived from interphase chromatin in two ways: (1) from the relative distance measured between pairs of probes and (2) from the order of red and green dots in nuclei hybridized with three or more probes. The second approach is more simple and direct but reveals order without distance information. Interphase mapping requires efficient hybridization and detection of DNA sequences in G_1 interphase nuclei. This can be achieved easily with 20–40 kbp probes, although our results using the plasmid st14c indicate that as little as 9 kbp may be sufficient. G_1 nuclei are the preferred target for deriving map information from hybridization sites. A doubling of the number of hybridization sites during DNA replication (B. J. Trask, unpublished observations) can be a source of confusion for mapping, especially if the sites of two probes are labeled with the same fluorochrome and one of the two is labeled with low efficiency. Detection of probes in different colors, which ensures that both probes of a pair have hybridized to the same nucleus, can reduce this confusion. Recently developed double-band pass filters make it possible to view both FITC and Texas red simultaneously without image shift. We prefer to label small or weakly hybridizing probes with biotin rather than with digoxigenin because the signal intensity of biotinylated probes can be amplified by alternating layers of avidin–Texas red and biotinylated anti-avidin antibody (Pinkel et al. 1986).

FISH mapping has provided a more complete map of a portion of Xq28 than was achievable using genetic or PFGE analysis alone. The map of Xq28 derived from interphase measurements is shown in figure 1. The map covers 2–3 Mbp, as estimated by PFGE analysis (Patterson et al. 1988, 1989*a*, 1989*b*; Kenwrick and Gitschier 1989). The FISH mapping approach was validated by the following findings: (1) The interphase map for the F8/CV complex corresponds with the PFGE restriction map for this 810-kb region (Kenwrick and Gitschier 1989). (2) The order, in interphase, of c1A1–st14c–(F8/CV) is consistent with recombination data (Patterson et al. 1989*a*). The proximal position, on metaphase chromosomes, of c1A1 and st14c relative to F8/CV probes serves to orient the map relative to the centromere and is consistent with genetic mapping (Patterson et al. 1989*a*). Given this validation, interphase mapping was used to orient the CV gene complex with respect to G6PD. This orientation should be considered tentative until it is confirmed by other physical mapping approaches. In light of a previous finding (Aubourg et al. 1990), it does suggest that the locus for adrenoleukodystrophy may lie between the telomere and the red pigment gene. In nine of nine mapping experiments in interphase chromatin, we have also shown that F8 is closer to c1A1 than is CV. Assuming random folding of chromatin over the distance spanning c1A1, F8, and CV, we conclude that F8 is proximal to CV. This order agrees with the combined results of genetic and PFGE mapping (Kenwrick and Gitschier 1989; Patterson et al. 1989*a*). Only in metaphase chromosomes does CV appear proximal to F8 (see below).

In the present paper, we have shown that the slope of the relationship between interphase and genomic distance is greatest—and thus most informative for determining probe order—between 100 kbp and 600–800 kbp. Lawrence et al. (1990) have also shown a correlation between interphase and genomic distance by using probes within 1 Mbp in the dystrophin gene on chromosome X. The degree of chromatin condensation and the theoretical limitations of light microscopy (0.24 μm in our system) limit the use of interphase mapping below ≈50 kbp (Trask et al. 1989). Interphase distances measured between c1A1 and probes from the F8/CV complex appear to increase gradually beyond distances estimated as >1 Mbp, but with a lower slope than in the 50–600-kbp range. We have previously observed that interphase distance continues to increase between sequences 50–90 Mbp apart (4–8 μm) (Trask et al. 1989). On the basis of the observed relationship between interphase and linear distance, reference probes can be chosen to order other probes. Order of three probes is best determined if they are equally spaced. For example, 18b41 and fr7 map 190 kbp apart and flank the CV genes. We chose to order these probes relative to G6PD, which maps ⩽360 kbp from CV, rather than relative to more proximal probes.

Absolute distance is more difficult to determine from interphase measurements than is relative order. This is due to several factors. Distance measurements between any two cosmids are variable, resulting in large standard errors of the mean (SEMs). The relationship between interphase and linear distance is not necessarily monotonic. Further, at distances >600 kbp the slope of the relationship is low and may vary between different nuclear preparations. Until the variables causing these differences can be identified, genomic distance between two probes should be inferred by comparing the measured distance between them with distances measured, on the same nuclear preparation, between probes of known map distance. For these reasons, we have used only the mean interphase distance between c1A1 and st14c (0.9 μm) to infer the map distance between them (400–600 kbp). We are unable to use inter-

phase distance to estimate the distance between c1A1 and F8, other than to confirm that it is >1 Mbp.

It is not yet known whether the absolute interphase distances observed in Xq28 can be generalized for other regions of the genome (telomeric vs. interstitial regions, Giemsa-dark vs. Giemsa-light bands, and actively transcribed vs. noncoding regions). Until generality is documented, the interphase distance between two unknown probes should be compared with distances measured for other probes, whose map position is known, from the same chromosomal region. One indication that interphase distance may be similar for different regions is that our previous data from the 250-kbp region around the CHO DHFR gene (Trask et al. 1989) are similar, in terms of both slope and absolute distances measured, to the Xq28 data.

The packing order in hybridized interphase chromatin can be calculated from the measured proximity of probe sites. The average compaction of the DNA between sequences 400 kbp apart in the F8/CV cluster is ≈100 times that of an unfolded DNA helix (interphase measurement is 0.72 μm × $4/\pi$ [maximal correction for underestimating, by the projection of hybridization-site positions onto the observation plane, the actual distances in a sphere] = 0.92 μm; linear DNA helix is 250 kbp × 0.34 μm/kbp = 85 μm). This result suggests that DNA between sites 400 kbp apart is only several fold more condensed than the 30-nm fiber seen in electron microscopic images (packing ratio ≈40 [Nelson et al. 1986]). We presume that the packing order increases between more distantly spaced sequences as the number of folds and levels of organization between them increases.

Our data show that interphase mapping has at least a 10-fold-higher resolution for ordering sequences than does two-color metaphase mapping (50–100 kbp vs. >1 Mbp). The relative order in metaphase of all probe pairs tested whose separation exceeds 1 Mbp is consistent with other genetic, PFGE, and interphase results. Even so, despite an estimated distance of 2–3 Mbp between the F8/CV complex and c1A1 or st14c, probes from the F8/CV complex appear distal to c1A1 or st14c on only 59%–70% of chromatids. Thus, even over these distances, it is necessary to score a number of chromatids to derive probe order with statistical confidence. Metaphase mapping fails to correctly order the more closely spaced pairs in Xq28, c1A1–st14c, and F8-CV. As noted above, interphase distance predicts that c1A1 and st14c are 400–600 kbp apart. F8 and CV are ≤810 kbp apart (Kenwrick and Gitschier 1989). These results suggest that the way chromatin is packed in metaphase may disrupt the linear order of closely spaced sequences. Our metaphase results are in agreement with the results of Lichter et al. (1990), who mapped chromosome 11 probes relative to the telomere. Their map accuracy was 2% of the total chromosome length, corresponding to 3 Mbp. The order of F8 and CV relative to the telomere in metaphase chromosomes is inverted relative to interphase results shown here and to PFGE mapping results (Kenwrick and Gitschier 1989). This apparent inverted order may be an artifact of chromatin folding near the telomere in metaphase. This hypothesis is substantiated by the observation using FISH that an oligomer specific for human telomeres appears proximal to the visible end of metaphase chromosomes (Moyzis et al. 1988).

The validity of the Xq28 map derived from interphase distance will be tested by comparing interphase distances measured between cosmids mapped within other regions spanning 3 Mbp in the human genome (e.g., around the MHC locus on chromosome 6 [Carroll et al. 1987] and around the Huntington disease locus on chromosome 4 [Bućan et al. 1990]). We are continuing to use interphase mapping to identify new sequences from Xq28 and to construct a long-range map of this region. This approach, together with other physical mapping methods (Warren et al. 1987; Ludecke et al. 1990; Wada et al. 1990), should unequivocally order markers and aid the identification of disease loci in this genetically important region.

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